Journal of Infectious Diseases Advance Access published December 7, 2012

Aspergillus fumigatus activates thrombocytes by secretion of soluble compounds

Cornelia Speth, Magdalena Hagleitner, Helmut W. Ott, Reinhard Würzner, Cornelia Lass-Flörl, and Günter Rambach

Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria

Address correspondence and page proofs to: Dr. Cornelia Speth, Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Fritz-Pregl-Straße 3, A-6020 Innsbruck, Austria, Phone: +43 512 9003 70705, Fax: +43 512 9003 73700, E-mail: cornelia.speth@i-med.ac.at

Abstract

In the course of invasive aspergillosis, platelets might be involved in immune defense, but also contribute to pathology of the disease. We tested the hypothesis that *Aspergillus* secretes factors that influence activity and functionality of thrombocytes. Platelets were incubated with medium wherein *A. fumigatus* was grown. This fungal culture supernatant potently stimulated thrombocytes time- and dose-dependently, inducing release of alpha and dense granules, membrane alterations, aggregation, and formation of microparticles. Fungus-induced platelet activation could be confirmed in vivo: thrombocytes from mice infected with *A. fumigatus* showed a higher activation level than platelets from non-infected animals. Two stimulating components in the fungal culture supernatant were identified: a fungal serine protease and the mycotoxin gliotoxin.

Activation of platelets by fungal factors stimulates antifungal functions: platelets gain the capacity to interact with foreign particles, and they become able to inhibit fungal growth, thus supporting the host immune network. However, some consequences of platelet activation might also be harmful, including excessive inflammation and induction of thrombosis. These findings imply that measuring platelet activation in patients might be an interesting diagnostic parameter.

Introduction

Aspergillus fumigatus represents one of the most frequent causes of invasive mycoses in immunosuppressed individuals (1;2). A more profound knowledge of pathogenesis and interactions between fungus and immune cells might help to develop new therapeutic approaches.

Emerging evidence assigns a crucial role to thrombocytes as immune mediators that participate in the innate immune response against bacteria, viruses and fungi (3-6). Two roles can be attributed to platelets: (a) direct attack of the invading pathogen and (b) interaction with and stimulation of innate and adaptive immune elements. A prerequisite for these functions is the activation of the thrombocytes, which is a multistep process that includes release of alpha and dense granules, membrane alterations, microparticle formation, and aggregation (7). However, excessive activation of the thrombocytes might promote thrombosis, a hallmark of an invasive Aspergillus infection, and thus contribute to the pathology of the disease (8;9).

Platelet activation after fungal infection might occur directly by contact with the fungal surface, and indirectly by secreted fungal factors. The first mechanism is restricted to sites of fungal infection, whereas secreted substances might affect platelets also from a distance to the fungal hyphae, thus putatively playing an even more important physiological role. It is well known that *Aspergillus* secretes a broad spectrum of different factors during growth, including enzymes, mycotoxins, and other metabolites (10-12). In the present study, we tested potential effects of secreted fungal factors on activation and antimicrobial capacity of thrombocytes.

Materials and Methods

Antibodies and chemicals

Antibodies and Annexin V were from BioLegend (San Diego, CA, USA), protease inhibitor cocktail was from Roche Diagnostics (Penzberg, Germany). All other protease inhibitors, FITC (Fluorescein isothiocyanate), gliotoxin, glucose, glutamine and arginine were purchased from Sigma (Sigma Aldrich, St. Louis, USA). Calcofluor white (Fungi-Fluor^{TM} Kit) was from Polysciences Inc.

Aspergillus fumigatus isolates and fungal supernatant preparation

A clinical isolate of *A. fumigatus* (A22) from a lung biopsy of an immunosuppressed patient with respiratory insufficiency was used for the majority of experiments. Nine further clinical isolates, derived from patients with cerebral and pulmonary aspergillosis, as well as six environmental isolates were compared for their capacity to secrete platelet-activating factors. Moreover, ATCC46645 (C1), and the corresponding knockout mutant C1ΔgliP that is deficient in gliotoxin peptide synthetase (GliP) (13), were tested.

To obtain the fungal supernatant (SN), 10^4 conidia of the *A. fumigatus* strains were inoculated in 500µl of RPMI medium (Invitrogen, Life Technologies) at 37°C. After 2 days, the fungus was removed by centrifugation and filtration, applying Spin-X filters (Corning Life Sciences, USA). The fungal supernatant was used either freshly or frozen at -20°C for further disposal.

Preparation of platelet-rich plasma and of whole-blood samples

All studies were approved by the local ethics committee. Blood was obtained with informed consent from healthy volunteers. Venous blood was taken with a trisodium citrate blood collection system (Sarstedt, Nümbrecht, Germany); platelet-rich plasma (PRP) was prepared from whole-blood by centrifugation at 135g for 15 min at room temperature.

Platelets were incubated in medium, 0.1U thrombin (Sigma Aldrich), or with fungal supernatant for up to 90 minutes at 37°C. In all experiments, incubation of platelets in RPMI medium, which does not induce any activation, represents the negative control, indicating the background of quiescent cells. To demonstrate that thrombocytes were not affected by preparation and are capable to be activated, thrombin was used as positive control. In some samples, the fungal SN was pre-incubated with protease inhibitors. PI is a cocktail containing inhibitory compounds against different classes of proteases. Other used protease inhibitors were E64 (cysteine proteases) and pepstatin (aspartyl proteases); chymostatin and 4-(2-Aminoethyl)-benzensulfonylfluorid (AEBSF) inhibit serine proteases. Furthermore, purified gliotoxin was used in some experiments.

Analysis of alpha-granules and dense granule release during platelet activation

Platelet activation by fungal supernatant or by thrombin was examined by detection of different platelet activation markers, using a FACSCanto flow cytometer (Becton Dickinson, San Diego, USA). Thrombocytes were gated by fluorescein isothiocyanate (FITC)-conjugated antibodies against CD41 or CD42a, which are antigens present on all platelets. Activation-induced secretion of alpha-granules and dense granules was quantified by measuring CD62P (P-selectin) or CD63, respectively, on the surface with specific antibodies. To analyze ATP release, thrombocytes were incubated with medium, thrombin, or increasing concentrations (v/v) of fungal supernatant. After 60 min, the culture medium was harvested, and the ATP content was quantified by luminometry, applying a commercial kit (Promega) according to manufacturer's instructions, and a luminometer (BioRad).

Analysis of membrane alterations and microparticle formation

Phosphatidylserine (PS), which is exposed on the surface of activated platelets (14), was detected by binding of Annexin V. Thrombocytes were incubated with FITC-conjugated

Annexin V according to the manufacturer's instructions (BioLegend), and subsequently analyzed by FACS.

To determine microparticle formation, platelets were incubated with medium or fungal SN for 90 min. Afterwards, newly formed microparticles positive for the platelet marker CD41 were gated in the samples according to their size, using the FACSCanto cytometer.

Aggregation assay

This feature of activated platelets was measured with the Multiplate® analyzer (Verum Diagnostica, Munich, Germany). Platelet-rich plasma was pre-incubated at 37°C, followed by addition of either fungal supernatant or 32µM thrombin receptor activating peptide (TRAP-6) (Verum Diagnostica). Aggregation was continuously recorded for 30 minutes. The impedance change is expressed in arbitrary „Aggregation Units"(AU) and plotted against the time. Platelet aggregation is quantified as area under the curve (AUC) in units (U).

Animal experiments

Experiments were conducted using 7-week-old female Balb/c mice (Charles River Laboratory). Mice were treated in accordance with the guidelines of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" and the Austrian law. Animal experiments were approved by the ethics committee of the Austrian Federal Ministry of Science and Research (BMWF).

Six animals per experimental group were used. Half of the mice were infected intravenously with 2 x 10^6 conidia of *A.fumigatus*. Blood was taken at day 3, and thrombocytes were analyzed for exposure of CD62P on their surface.

Interaction of thrombocytes with particles and inhibition of fungal growth

Platelet-rich plasma was labeled with antibodies against CD42a (BD Pharmingen). A tenfold amount of either FITC-labeled latex beads (Sigma) or FITC-labeled conidia was added. After 60 min, cells were fixed with 1% formalin, and CD42a-positive thrombocytes that were also positive for FITC were quantified by flow cytometry.

To analyze the effect of thrombocytes on fungal growth, conidia of *A.fumigatus* were incubated with medium, quiescent thrombocytes, or thrombocytes preincubated with fungal SN (platelets:conidia = 1:10); germination of the conidia was observed in a life-time microscope (Nikon, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed with unpaired t-test, using the Graph-prism software.

Results

Thrombocytes react on contact with *A. fumigatus*-derived secretory factors with release of alpha granules

The reaction of platelets on the presence of *A. fumigatus*-derived culture supernatant (SN) was evaluated by incubation of thrombocytes with fungal SN, followed by measurement of the activation marker CD62P. CD62P is present in the alpha granules and appears on the cell surface after fusion of these granules. The soluble factors released by *Aspergillus fumigatus* induced a strong increase in the membrane exposure of CD62P (Fig. 1A); The extent was similar to that after addition of thrombin, which is one of the strongest platelet activators. Time-dependence of the *Aspergillus*-induced thrombocyte activation is shown in Fig. 1B. A highly significant accumulation of CD62P on the surface is visible 5 min after addition of the fungal SN, and it constantly increases for up to 90 min. To study the dose-dependence of

platelet stimulation, different volumes of the fungal SN (%v/v) were added to the cells (Fig. 1C). Significant rise of CD62P signal on the cells was visible already with 2% SN as final volume of the sample.

Thrombocytes undergo a broad-spectrum activation program after contact with *A. fumigatus*-derived secretory factors

To evaluate whether fungal SN also triggers fusion of platelet dense granules with the plasma membrane, CD63 surface exposure and ATP release were checked (Fig. 2A,B). A concentration of 1% (v/v) of fungal supernatant was sufficient to stimulate significant release of dense granules, as shown by appearance of CD63 on the surface (Fig. 2A); higher concentrations of *Aspergillus* SN triggered further upregulation of CD63. Furthermore, ATP, a substantial content of the dense granules, is released into the medium to a significant extent (Fig. 2B).

As activated platelets have been described to undergo membrane alterations, the exposure of phosphatidylserine after addition of the fungal SN was quantified by binding of Annexin V. Significant increase of Annexin V binding could be proven with 20% (v/v) Aspergillus SN (Fig. 2C). Enhanced binding could be measured also with lower concentrations of SN, with 1% (v/v) as lower limit (unpublished data).

Since budding of microparticles from activated platelets represents an important process to potentiate inflammatory processes, we analyzed their generation. Significant amounts of microparticles appeared after addition of 1% (v/v) fungal SN (Fig. 2D; dot blots and graph); higher concentrations did not further increase their formation.

Aggregation of the activated platelets might play a role in fungus-induced thrombosis. For that reason, the aggregation of the thrombocytes under influence of fungal secretory factors was monitored three times, using platelets derived from different blood donors. A representative experiment and the corresponding quantification of aggregation as area under

the curve (AUC) are shown in Fig. 3. A strong reaction could be triggered with the *Aspergillus* SN, compared to the control cells incubated only with medium. Only TRAP, a peptide known to be a potent inducer of aggregation, induced a higher effect.

Fungus-induced thrombocyte activation also occurs in vivo

We aimed to confirm that fungus-induced thrombocyte activation also occurs in vivo and thus might take place in infected patients. Therefore, we compared the effect of fungal secretory factors on thrombocytes in platelet-rich plasma (PRP) with that on platelets present in a whole-blood sample. This approach enabled us to check whether this reaction also takes place in the interplay of thrombocytes with other immune cells and plasma-derived soluble factors. As shown in Fig. 4A, thrombocytes could be activated by addition of fungal SN both in PRP and in whole blood. In both preparations, the reaction of platelets could be triggered with concentrations of 1% (v/v) *Aspergillus* SN or higher. The maximal stimulation of the thrombocytes in whole-blood needed higher volumes of the fungal secretory factors than in PRP, probably due to the interaction of these factors also with other blood cells (Fig.4A). Next, we compared environmental versus patient-derived isolates of *A. fumigatus* for their capacity to stimulate platelets. As shown in fig. 4B, the fungal supernatants of patient-derived isolates tendentially activate thrombocytes to a higher extent than the secreted factors of isolates found in the environment. However, the difference between the two groups did not reach significance.

To further approach the in vivo situation of invasive aspergillosis, we performed an animal experiment. Mice were either mock-treated or intravenously infected with *Aspergillus fumigatus,* and blood samples were taken from the animals at day 3. The extent of platelet activation was analyzed with CD62P as relevant marker. Animals infected with *A.fumigatus* showed significantly more CD62P on the surface of their thrombocytes than the mock-treated control animals (Fig. 4C).

A fungal serine protease and gliotoxin contribute to the thrombocyte-activating effect of fungal supernatant

The fungal supernatant represents a complex mixture of different compounds, secreted by *A. fumigatus* during growth. We tried to get some hints, which substance(s) in the SN might be responsible for thrombocyte activation. Since the presence of nutrients in the medium regulates the synthesis and secretion of fungal factors, we supplemented the medium with glucose as carbon source or arginine or glutamine as nitrogen source. While glucose did not modulate the presence of platelet-activating factors in the supernatant, supplementation with amino acids reduced the stimulating effect (Fig. 5A).

Since nitrogen is known to regulate the synthesis of fungal proteases, we investigated whether proteases in the fungal SN might mediate platelet activation via protease-activated receptors on thrombocytes. Preincubation of the *A. fumigatus* SN with a cocktail of protease inhibitors strongly reduced its capacity to stimulate platelets, as shown by CD62P quantification (Fig. 5B). To further determine which class of proteases is involved, a panel of more specific protease inhibitors was used. E64 is a highly selective inhibitor of cysteine proteases, but was unable to interfere with the platelet-activating effect of the fungal SN. Similarly, pepstatin, which inhibits aspartic proteases, could not abolish the platelet stimulation by fungal SN. In contrast, the inhibition of serine proteases by AEBSF or chymostatin significantly down-modulated the ability of the fungal SN to activate thrombocytes, thus indicating the contribution of one or several serine protease(s) (Fig. 5B).

Mycotoxins are generated and released during fungal growth, with gliotoxin as the most prominent representative . To determine whether gliotoxin might be involved in stimulation of thrombocytes, we used an *Aspergillus fumigatus* mutant (C1ΔgliP) lacking an enzyme for gliotoxin synthesis. The corresponding wild-type (wt) strain C1 was used for control. The wt strain produced platelet-activating compounds to a similar extent as the *A. fumigatus* strain we

used for all former experiments. However, the supernatant of the ΔgliP mutant lacked nearly all capacity to trigger thrombocyte activation (Fig. 5C). Further evidence for a principle role of this mycotoxin in platelet activation was supplied by an additional experiment, where the effect of the whole fungal SN was mimicked by purified gliotoxin (Fig. 5D). Gliotoxin concentrations as low as 3nM were sufficient to induce thrombocyte activation, as demonstrated by CD62P increase; 30nM gliotoxin showed a similar effect as the tested fungal supernatant. Higher amounts of gliotoxin up to 1000nM further enhanced the CD62P signal.

SN-stimulated thrombocytes show enhanced interaction with fungal pathogens

We aimed to clarify whether thrombocytes can fulfill antimicrobial functions and interact with the fungus after activation by *A. fumigatus* supernatant. Fungal hyphae were stained with calcofluor white; and CD42a-labeled platelets, either mock-treated or pre-activated with fungal SN, were added. Microscopic analysis clearly showed that quiescent thrombocytes bound to the hyphae to a moderate extent (Fig. 6A); in contrast, the pre-stimulated platelets revealed a very high level of interaction with the hyphae (Fig. 6B).

We further quantified the stickiness of SN-activated platelets to foreign particles by FACS analysis, mimicking a putative pathogen by using FITC-labeled latex beads. No differentiation between adhesion and internalization can be made by this method. As shown in Fig. 6C, the SN-triggered platelets bind or even ingest the beads in a dose-dependent manner. The interaction with the larger *A. fumigatus* conidia is much lower, but still significantly increased in SN-stimulated compared to unstimulated thrombocytes (Fig. 6D).

SN-stimulated thrombocytes interfere with fungal germination and growth

The inhibition of fungal germination and proliferation by activated thrombocytes was tested. Addition of quiescent platelets to *A.fumigatus* conidia delayed germination and hyphal elongation as shown in Fig. 7A. However, the presence of platelets that were activated by

fungal SN nearly completely suppressed any germination of the *Aspergillus* conidia (Fig.7A).The SN of the ΔgliP mutant was much less effective to induce the fungal growth-inhibitory capacity of thrombocytes than the corresponding wild-type strain (Fig. 7B). Addition of the protease inhibitor AEBSF, which had been demonstrated to interfere with platelet activation to the supernatant did not affect platelet activation, indicating that proteases do not participate in that effect (Fig. 7C).

Discussion

Thrombocytes were recently discovered to be part of the innate immunity and to harbor antimicrobial functions (5;15-17). The direct physical contact with fungal conidia or hyphae has been described to stimulate platelets (18-20). We could show that they also sense the presence of fungus-derived soluble factors. The subsequent stimulation and antimicrobial activity are therefore not limited to the proximity of fungal hyphae, but can occur all over the body in patients with invasive aspergillosis and thus may considerably affect the course of disease. However, the possibility of a disseminated thrombocyte reaction implies that platelets could also contribute to excessive inflammation and thrombosis (8).

The evidence that SN-induced thrombocyte activation also occurs in whole-blood samples, strongly asks for a deeper insight into the role of thrombocytes in infected patients. Fungal isolates derived from infected patients are by tendency more potent to activate platelets than environmental isolates. The data of a mouse model of invasive aspergillosis, showing the activation status of thrombocytes to be higher in infected than in non-infected animals, further underlines the in vivo relevance of our results, although this model allowed no differentiation between thrombocyte activation by direct contact with fungi and stimulation by released fungal factors.

Thrombocytes sense the presence of secreted fungal factors by an as yet unknown mechanism. The complex composition of the fungal supernatant suggests that more than one cellular receptor might be involved. The reaction of the thrombocytes comprises the complete arsenal of activation steps: release of platelet alpha and dense granules, membrane alteration, aggregation, and microparticle formation (14;21;22). The precise kinetics and dose-dependence of the different processes, which characterize the activation process, vary, particularly between alpha granules (CD62P) and dense bodies (CD63, ATP release). A putative explanation for that phenomenon might be the interaction with the actin cytoskeleton that has been described to differentially regulate these two types of granules (23). The risk of an excessive platelet reaction is increased by the fact that activation produces mediators that might further stimulate other platelets and immune cells. Microparticles, which are budded after contact of the platelets with fungal SN, represent well known mediators of inflammation, and their number correlates with the severity of e.g. septic shock (24-27). Furthermore, released ADP and ATP can activate other thrombocytes, thus resulting in an overflow of the inflammatory reaction. In addition, thrombocytes were described to store chemokines and cytokines in their granules; therefore, the triggering of granule release, as demonstrated in this paper, might also result in enhanced communication with and activation of other immune cells (8;15).

To simulate the situation in vivo, where fungi produce and secrete a broad spectrum of different factors, we used the complete fungal supernatant for our experiments. More detailed studies revealed that activation is a multifactorial process with an *Aspergillus*-derived serine protease and the mycotoxin gliotoxin as participating compounds.

Fungi are known to secrete a large variety of different proteases (11); some of them might be hypothesized to mimic mammalian serine proteases, which are known to be involved in regulation of haemostatic, inflammatory, and thrombotic processes (28). Mammalian serine proteases (e.g. thrombin) bind to protease-activated receptors (PARs) and induce

transmembrane signaling (28). This mechanism, designed to link tissue injury to cellular responses, might also be triggered by *Aspergillus*-derived serine proteases.

Beside the proteases, we could demonstrate a role of gliotoxin in platelet stimulation and thus hypothesize that it might considerably influence the course of invasive aspergillosis. Gliotoxin, a member of the epipolythiodioxopiperazine class of fungal toxins, is produced predominantly by *A. fumigatus* and has a toxic and immunosuppressive effect on granulocytes, monocytes, and microglia (29;30). Gliotoxin can suppress phagocytosis, inflammatory response and cytokine production, mainly via generation of reactive oxygen species and blockage of NF-κB (29;31).

Platelets are more resistant against the toxicity of gliotoxin than other immune cells, and concentrations of up to 1000nM gliotoxin still induced activation. This might be due to the fact that thrombocytes lack transcriptional capacity and thus are insensitive for NF-κB blocking. Thus, under conditions where most other immune cells are functionally suppressed, platelets can still undergo activation and inhibit fungal germination and hyphal elongation. The gliotoxin concentrations of 3-1000nM, that trigger thrombocyte activation, are also reached in vivo. Serum samples derived from patients with invasive aspergillosis contain between 508 to 2405nM gliotoxin (32).

Stimulated by fungal SN, thrombocytes gain a substantial antimicrobial function: they efficiently bind to fungal hyphae and interact with or even internalize foreign particles. The binding to hyphae can by hypothesized to be an important step in the immune defense against *Aspergillus*, since platelets store antimicrobial peptides (AMPs) (6) and serotonin, which also harbors antifungal activity (33). Binding of platelets to hyphae under the influence of the fungal SN might help to directly target the release of AMPs and serotonin to the site of fungal presence. Interaction of activated platelets with conidia is much lower than with hyphae, probably due to the different surface composition. Furthermore, our results show that contact with fungal SN enables thrombocytes to interact in general with foreign particles (e.g. latex

beads), which is a prerequisite for their phagocytic capacity (34). Conidia, that are larger than latex beads, show moderate interaction with the platelets.

The second immunological function that is activated by fungal SN is the ability of platelets to inhibit fungal germination. Interestingly, fungal proteases seem to play no role for the induction of this platelet function, whereas gliotoxin is central for its stimulation. This finding supports the hypothesis that the modulation of platelets by the fungal supernatant is a multifactorial process.

A putative consequence of our study might be the identification of those invasive aspergillosis patients that are at particular risk for the development of thrombosis. Patients with high levels of CD62P on their platelets and strong Annexin V binding might be defined to be pre-disposed for thrombus formation, with improved antithrombotic prophylaxis as putative consequence. Further studies in vitro and in vivo will help to work towards this aim.

Funding

This work was supported by the Medizinischer Forschungsfonds Tirol [MFF; Project Nr. 212].

Acknowledgements

We thank Dr. Claudio Kupfahl for the kind provision of the *Aspergillus fumigatus* ΔgliP mutant and the corresponding wild-type strain C1. All authors declare that there are no conflicts of interest, and have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

Footnote page

Conflict-of-interest statement: All authors hereby declare that there is no commercial or other association that might pose a conflict of interest.

Funding statement: This study was supported by the Medizinischer Forschungsfonds Tirol (MFF; Project Nr. 212).

Meetings at which parts of the information have been presented:

- 5th Trends in Medical Mycology (TIMM); October 2011; Valencia, Spain (Abstract O3.6)
- 5th Advances Against Aspergillosis (AAA); January 2012; Istanbul, Turkey (Abstract P66)

Correspondence to: Dr. Cornelia Speth

Division of Hygiene and Medical Microbiology

Innsbruck Medical University

Fritz-Pregl-Str. 3, A-6020 Innsbruck, Austria.

Tel: +43 512 9003-70705

Fax: +43 512 9003-73700

e-mail: cornelia.speth@i-med.ac.at

Alternate corrresponding author: Dr. Günter Rambach

Division of Hygiene and Medical Microbiology

Innsbruck Medical University

Fritz-Pregl-Str. 3, A-6020 Innsbruck, Austria.

Tel: +43 512 9003-70705

Fax: +43 512 9003-73700

e-mail: guenter.rambach@i-med.ac.at

Reference List

(1) Denning DW. Invasive aspergillosis. Clin Infect Dis **1998 Apr**;26(4):781-803.

(2) Kullberg BJ, Lashof A.M.L. Epidemiology of opportunistic invasive mycoses. Eur J Med Res **2002**;7:183-91.

(3) Leslie M. Cell biology. Beyond clotting: the powers of platelets. Science **2010 Apr 30**;328(5978):562-4.

(4) Li Z, Yang F, Dunn S, Gross AK, Smyth SS. Platelets as immune mediators: their role in host defense responses and sepsis. Thromb Res **2011 Mar**;127(3):184-8.

(5) Semple JW, Italiano JE, Jr., Freedman J. Platelets and the immune continuum. Nat Rev Immunol **2011 Apr**;11(4):264-74.

(6) Yeaman MR, Bayer AS. Antimicrobial peptides versus invasive infections. Curr Top Microbiol Immunol **2006**;306:111-52.

(7) Jurk K, Kehrel BE. Platelets: physiology and biochemistry. Semin Thromb Hemost **2005**;31(4):381-92.

(8) Wagner DD, Burger PC. Platelets in inflammation and thrombosis. Arterioscler Thromb Vasc Biol **2003 Dec**;23(12):2131-7.

(9) Lewis RE, Kontoyiannis DP. Invasive aspergillosis in glucocorticoid-treated patients. Med Mycol **2009**;47 Suppl 1:S271-S281.

(10) Ben-Ami R, Lewis RE, Leventakos K, Kontoyiannis DP. Aspergillus fumigatus inhibits angiogenesis through the production of gliotoxin and other secondary metabolites. Blood **2009 Dec 17**;114(26):5393-9.

(11) Monod M, Capoccia S, Lechenne B, Zaugg C, Holdom M, Jousson O. Secreted proteases from pathogenic fungi. Int J Med Microbiol **2002 Oct**;292(5-6):405-19.

(12) Reijula K, Tuomi T. Mycotoxins of aspergilli: exposure and health effects. Front Biosci **2003 May 1**;8:s232-s235.

(13) Kupfahl C, Heinekamp T, Geginat G, et al. Deletion of the gliP gene of Aspergillus fumigatus results in loss of gliotoxin production but has no effect on virulence of the fungus in a low-dose mouse infection model. Mol Microbiol **2006 Oct**;62(1):292-302.

(14) Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur J Biochem **1982 Feb**;122(2):429-36.

(15) Nurden AT. Platelets, inflammation and tissue regeneration. Thromb Haemost **2011 May**;105 Suppl 1:S13-S33.

(16) von HP, Weber C. Platelets as immune cells: bridging inflammation and cardiovascular disease. Circ Res **2007 Jan 5**;100(1):27-40.

(17) Zander D, Klinger MH. The blood platelets contribution to innate host defense - what they have learned from their big brothers. Biotechnol J **2009**;4:914-26.

(18) Perkhofer S, Kehrel BE, Dierich MP, et al. Human platelets attenuate Aspergillus species via granule-dependent mechanisms. J Infect Dis **2008 Oct 15**;198(8):1243-6.

(19) Perkhofer S, Kainzner B, Kehrel BE, Dierich MP, Nussbaumer W, Lass-Florl C. Potential antifungal effects of human platelets against zygomycetes in vitro. J Infect Dis **2009 Oct 1**;200(7):1176-9.

(20) Rodland EK, Ueland T, Pedersen TM, et al. Activation of platelets by Aspergillus fumigatus and potential role of platelets in the immunopathogenesis of Aspergillosis. Infect Immun **2010 Mar**;78(3):1269-75.

(21) McGregor L, Martin J, McGregor JL. Platelet-leukocyte aggregates and derived microparticles in inflammation, vascular remodelling and thrombosis. Front Biosci **2006**;11:830-7.

(22) Spangenberg P, Tschope D, Esser J, et al. Determination of platelet activation by assaying filamentous actin and detecting membrane alterations. Acta Histochem Suppl **1991**;41:187-92.

(23) Flaumenhaft R, Dilks JR, Rozenvayn N, Monahan-Earley RA, Feng D, Dvorak AM. The actin cytoskeleton differentially regulates platelet alpha-granule and dense-granule secretion. Blood **2005 May 15**;105(10):3879-87.

(24) Boilard E, Nigrovic PA, Larabee K, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science **2010 Jan 29**;327(5965):580-3.

(25) Horstman LL, Jy W, Ahn YS, et al. Role of platelets in neuroinflammation: a wide-angle perspective. J Neuroinflammation **2010**;7:10.

(26) Vasina E, Heemskerk JW, Weber C, Koenen RR. Platelets and platelet-derived microparticles in vascular inflammatory disease. Inflamm Allergy Drug Targets **2010 Dec**;9(5):346-54.

(27) Italiano JE, Jr., Mairuhu AT, Flaumenhaft R. Clinical relevance of microparticles from platelets and megakaryocytes. Curr Opin Hematol **2010 Nov**;17(6):578-84.

(28) Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost **2005 Aug**;3(8):1800-14.

(29) Kwon-Chung KJ, Sugui JA. What do we know about the role of gliotoxin in the pathobiology of Aspergillus fumigatus? Med Mycol **2009**;47 Suppl 1:S97-103.

(30) Speth C, Kupfahl C, Pfaller K, et al. Gliotoxin as putative virulence factor and immunotherapeutic target in a cell culture model of cerebral aspergillosis. Mol Immunol **2011 Sep**;48(15-16):2122-9.

(31) Gardiner DM, Waring P, Howlett BJ. The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. Microbiology **2005 Apr**;151(Pt 4):1021-32.

(32) Lewis RE, Wiederhold NP, Chi J, et al. Detection of gliotoxin in experimental and human aspergillosis. Infect Immun **2005 Jan**;73(1):635-7.

(33) Perkhofer S, Niederegger H, Blum G, et al. Interaction of 5-hydroxytryptamine (serotonin) against Aspergillus spp. in vitro. Int J Antimicrob Agents **2007 Apr**;29(4):424-9.

(34) Meseguer J, Esteban MA, Rodriguez A. Are thrombocytes and platelets true phagocytes? Microsc Res Tech **2002 Jun 15**;57(6):491-7.

Figure legends

Fig. 1: Release of alpha granules by thrombocytes, induced by culture supernatant of A. fumigatus

(A) Platelet-rich plasma (PRP) was incubated with RPMI medium, thrombin, or 20% (v/v) fungal supernatant (SN) from *Aspergillus fumigatus (A. fum.)* for 90 minutes. Exposure of CD62P was assessed by flow cytometry. (B) PRP was incubated with medium, thrombin, or 20% (v/v) fungal SN; CD62P was quantified at different time points. (C) PRP was incubated with medium, thrombin, or different volumes (v/v) of fungal SN; CD62P was measured after 90 min. Each experiment was repeated at least three times with triplicate samples. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 2: Different activation parameters of thrombocytes after incubation with culture supernatant of A. fumigatus

To assess the release of dense granules, thrombocytes were incubated with medium, thrombin, or increasing concentrations of *Aspergillus fumigatus* culture supernatant (SN) for 90 min; surface exposure of CD63 was measured by flow cytometry (A); secretion of ATP was quantified by luminometry (B). Cell membrane alteration of the platelets after incubation with 20% (v/v) fungal SN was analyzed as binding of FITC-labeled annexin V to exposed phosphatidylserine, using flow cytometry (C). The percentage of microparticles budded from thrombocytes was quantified by FACS; representative dot blots as well as the corresponding graph are shown (D). Each experiment was repeated at least three times with triplicate samples. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 3: Aggregation of thrombocytes stimulated by culture supernatant of A. fumigatus

Thrombocytes were incubated with either medium, 32µM TRAP-6 (thrombin receptor activating peptide), or 20% (v/v) of *A. fumigatus* supernatant (SN). Aggregation was continuously recorded for 30 minutes. The impedance change is expressed in arbitrary „Aggregation Units"(AU) and plotted against the time. Platelet aggregation is quantified as area under the curve (AUC) in units (U). The experiment was repeated three times with thrombocytes from different donors; a representative result is shown here.

Fig. 4: Platelet activation by A. fumigatus in different experimental models

(A) Platelet-rich plasma (PRP) or whole-blood samples were incubated for 90 min with medium, thrombin, or increasing concentrations (v/v) of *A. fumigatus* supernatant. Exposure of CD62P on CD41-positive thrombocytes was assessed by flow cytometry. (B) Supernatants (20% v/v) of patient-derived and environmental isolates of *A. fumigatus* were added to the platelets for 90 min. Exposure of CD62P on thrombocytes was studied by flow cytometry. These experiments (A,B) were repeated at least three times with triplicate samples. (C) Balb/c mice were infected intravenously with *A. fumigatus* conidia. Blood samples were taken at day 3 after infection, and the presence of CD62P on thrombocytes was assessed by FACS analysis. Each group consisted of six animals. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 5: Influence of protease and gliotoxin on thrombocyte activation by culture supernatant of A. fumigatus

(A) *A. fumigatus* was grown in medium or in medium supplemented with 1% glucose, 20mM glutamine, or 10mM arginine; supernatants were harvested after 2d. Platelets were incubated with thrombin, medium, supplemented media, or fungal supernatants at a final concentration of 20% (v/v). (B) Thrombocytes were incubated with medium, thrombin, or fungal culture

supernatant (SN) in a final concentration of 20% (v/v). The SN was partly pre-incubated with a protease inhibitor (PI) cocktail (onefold), or with the protease inhibitors E64 (40µM), pepstatin (1µM), AEBSF (2mM), or chymostatin (300µM), respectively. (C) Thrombocytes were incubated with medium, thrombin, or different fungal culture supernatants (SN), derived from the *Aspergillus fumigatus* wild-type strains A22, C1, or from the gliotoxin-deficient mutant C1ΔgliP, in a final concentration of 20% (v/v). (D) Platelets were incubated with medium, fungal supernatant (SN) or with increasing concentrations of purified gliotoxin. In all experiments, exposure of CD62P on the thrombocytes was measured by flow cytometry after 90 min of incubation. Each experiment was repeated at least three times with triplicate samples.* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 6: Interaction of activated platelets with beads, Aspergillus fumigatus hyphae and conidia

Aspergillus fumigatus hyphae were grown on a cover slip and stained with calcofluor white. Thrombocytes were stained with CD42a-PE, either incubated with medium (A) or with 20% (v/v) *A. fumigatus* supernatant (SN) (B) (original magnifications 100 x and 400 x, respectively). After 90 min, the samples were washed and assessed microscopically. The experiment was repeated three times; a representative result is shown here. (C) Thrombocytes were incubated with medium, thrombin, or increasing concentrations of fungal SN for 60 min. FITC-labeled beads (10:1) were added; after 30 min, the percentage of thrombocytes positive for FITC was evaluated by flow cytometry. (D) Thrombocytes were incubated with medium or 20% (v/v) *A. fumigatus* SN for 60 min; either FITC-labeled beads or FITC-labeled conidia (10:1) were added for 30 min, and the percentage of FITC-positive platelets was evaluated by FACS. These experiments (C,D) were repeated at least three times with triplicate samples.* $p<0.05$; ** $p<0.01$; *** $p<0.001$. Tc = thrombocytes

Fig. 7: Effect of thrombocytes on fungal germination and growth

(A) Conidia of *Aspergillus fumigatus* were co-incubated with medium, thrombocytes, or thrombocytes activated with 20% (v/v) *A. fumigatus* supernatant (SN). (B) Conidia of *Aspergillus fumigatus* were co-incubated with medium, or with thrombocytes activated with 20% (v/v) fungal SN derived from either *A. fumigatus* wild-type strain or the gliotoxin-deficient mutant C1ΔgliP. (C) Conidia of *Aspergillus fumigatus* were co-incubated with medium or thrombocytes activated with 20% (v/v) *A. fumigatus* supernatant (SN), either in presence or in absence of the protease inhibitor AEBSF. Germination of conidia and hyphal growth in the samples were observed in a lifetime microscope. The experiment was repeated three times, a representative result after 10h of co-culture is shown here. Original magnification is 200fold.